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Potato NPH3/RPT2-like protein StNRL1, targeted by a *Phytophthora infestans* RXLR effector, is a susceptibility factor

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E.M.G., H.M., P.C.B., Z.T., J.Z. and P.R.J.B. conceived of and designed the experiments. L.Y., L.G., P.C.B., H.M., M.A., Q.H., W.Z and S.N. performed the experiments. L.Y., H.M., M.A., Q.H., S.N., E.M.G. and P.R.J.B. analysed and interpreted the results. L.Y., H.M. and P.R.J.B. wrote the paper. P.R.J.B. and E.M. directed the project.

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Abstract

Plant pathogens deliver effectors to manipulate host processes. We know little about how fungal and oomycete effectors target host proteins to promote susceptibility, yet such knowledge is vital to understand crop disease. We show that either transient expression in *Nicotiana benthamiana*, or stable transgenic expression in potato (*Solanum tuberosum*), of *Phytophthora infestans* RXLR effector Pi02860 enhances leaf colonization by the pathogen. Expression of Pi02860 also attenuates cell death triggered by the *P. infestans* MAMP INF1, indicating that the effector suppresses pattern-triggered immunity (PTI). However, the effector does not attenuate cell death triggered by Cf4/Avr4 co-expression, showing that it does not suppress all cell death activated by cell surface receptors. Pi02860 interacts in yeast-2-hybrid assays with potato NPH3/RPT2-like 1 (NRL1), a predicted Cullin-3-associated ubiquitin E3 ligase. Interaction of Pi02860 *in planta* was confirmed by co-immunoprecipitation and bimolecular fluorescence complementation assays. Virus-induced gene silencing (VIGS) of *NRL1* in *N. benthamiana* resulted in reduced *P. infestans* colonization and accelerated INF1-mediated cell death, indicating that this host protein acts as a negative regulator of immunity. Moreover, whereas *NRL1* VIGS had no effect on the ability of *P. infestans* effector AVR3a to suppress INF1-mediated cell death, such suppression by Pi02860 was significantly attenuated, indicating that this activity of Pi02860 is mediated by NRL1. Transient overexpression of NRL1 resulted in suppression of INF1-mediated cell death and enhanced *P. infestans* leaf colonization, demonstrating that NRL1 acts as a susceptibility factor to promote late blight disease.

Introduction

Plant immunity is triggered by detection of conserved microbial molecules, microbe- or pathogen-associated molecular patterns (M/PAMPs), leading to pattern-triggered immunity (PTI), and by the detection of effectors, leading to effector-triggered immunity (ETI). Central to the successful colonisation of plants by phytopathogens is the delivery of effector proteins to suppress host immunity. Secreted effectors may act outside (apoplastic effectors) or be delivered inside (intracellular or cytoplasmic effectors) host cells to attenuate PTI or ETI (Jones and Dangl 2006). A broad range of host targets and activities have been elucidated for many bacterial type 3 secretion system effectors (Block and Alfano 2011; Deslandes and Rivas 2012; Dou and Zhou 2012). In contrast, less is understood about the effectors from filamentous phytopathogens: the fungi and oomycetes.

The oomycete *Phytophthora infestans* causes the devastating late blight disease of potato and tomato (Kamoun et al. 2014). Amongst the classes of candidate virulence determinants that have been identified are the RXLR effectors (Birch et al. 2006), which are delivered inside living plant cells (Whisson et al. 2007). Following the identification of the ubiquitin E3 ligase CMPG1 as a target of AVR3a (Bos et al. 2010), the targets and/or virulence activities of a small number of other *P. infestans* RXLRs have been revealed. AvrBlb2 prevents the secretion of a defence protease (Bozkurt et al. 2011), whilst AVR2 interaction with the putative phosphatase BSL1, involved in the brassinosteroid signal transduction pathway, facilitates recognition of the effector by the resistance protein R2 (Saunders et al. 2012). Effector Pi03192 interacts with NAC transcription factors, preventing their re-localisation from the endoplasmic reticulum to the host nucleus (McLellan et al. 2013). PexRD2 targets the host MAP3Kε, inhibiting signal transduction following perception of Cf-Avr4 from *Cladosporium fulvum* by tomato resistance Cf4 (King et al. 2014), whilst a number of effectors act redundantly to suppress flg22-mediated MAPK activation and early transcriptional changes (Zheng et al. 2014), implicating this signal transduction pathway also in response to unknown oomycete MAMPs. A K-homology class RNA binding protein, StKRBP1, which associates with RXLR effector Pi04089, provides the first evidence that *P. infestans* effectors manipulate host susceptibility factors to promote late blight disease (Wang et al. 2015). Pi04089 increases the abundance of

92 StKRBP1, a phenomenon which also occurs during the first 24 hours of *P. infestans*
93 infection. Overexpression of this RNA binding protein enhances leaf colonisation by
94 the pathogen (Wang et al. 2015). More recently, an RXLR effector from *P. infestans*
95 has been shown to target host PP1c isoforms. Rather than inhibiting these
96 phosphatases the effector forms unique holoenzymes with them to presumably
97 dephosphorylate key substrates in the plant nucleus, leading to enhanced
98 susceptibility (Boevink et al 2016). The PP1c isoforms can thus also be regarded as
99 susceptibility factors.

100 One of the key *P. infestans* MAMPs detected by solanaceous hosts is INF1, which
101 elicits BAK1-dependent cell death in the model host plant *Nicotiana benthamiana*
102 (Heese et al. 2007) and a range of *Solanum* species (Vleeshouwers et al. 2006).
103 Recently, a receptor that detects INF1 and other elicitins from a broad range of
104 oomycetes, termed ELR, has been cloned from *Solanum microdontum* (Du et al.
105 2015). Overexpression of ELR in the cultivated potato enhances disease resistance
106 to *P. infestans* (Du et al. 2015). INF1-mediated cell death can be suppressed by
107 AVR3a, either by inhibition or modification of CMPG1 activity (Bos et al. 2010; Gilroy
108 et al. 2011), and can be partially suppressed by RXLR effector Pi18215/SFI7, which
109 also inhibits flg22-mediated MAPK activation (Zheng et al. 2014).

110 In addition to CMPG1, another plant U-box (PUB) ubiquitin E3 ligase, PUB17, has
111 been shown to positively regulate immunity (Yang et al 2006). PUB17 functions in
112 the host nucleus to mediate both PTI, following perception of bacterial PAMP flg22,
113 and cell death triggered by co-expression of Cf4/Avr4. However, it is not involved in
114 INF1-triggered cell death (He et al 2015). In contrast to CMPG1 and PUB17, a
115 number of PUB E3 ligases have been shown to negatively regulate plant immunity
116 (Duplan and Rivas, 2014). PUB12 and PUB13 work in concert to attenuate PTI by
117 ubiquitinating the flg22 receptor FLS2, facilitating its degradation (Lu et al 2011).
118 PUB22, PUB23, and PUB24 also act to suppress immunity. In addition, NPR3 and
119 NPR4 contain Broad-Complex, Tramtrack and Bric-a-brac (BTB) domains that
120 facilitate interaction with cullin-3 E3 ligase. NPR3 and NPR4 negatively regulate
121 salicylic acid-mediated defences (Fu et al 2012). Ubiquitination is thus a post-
122 translational modification implicated in both positive and negative regulation of
123 immunity.

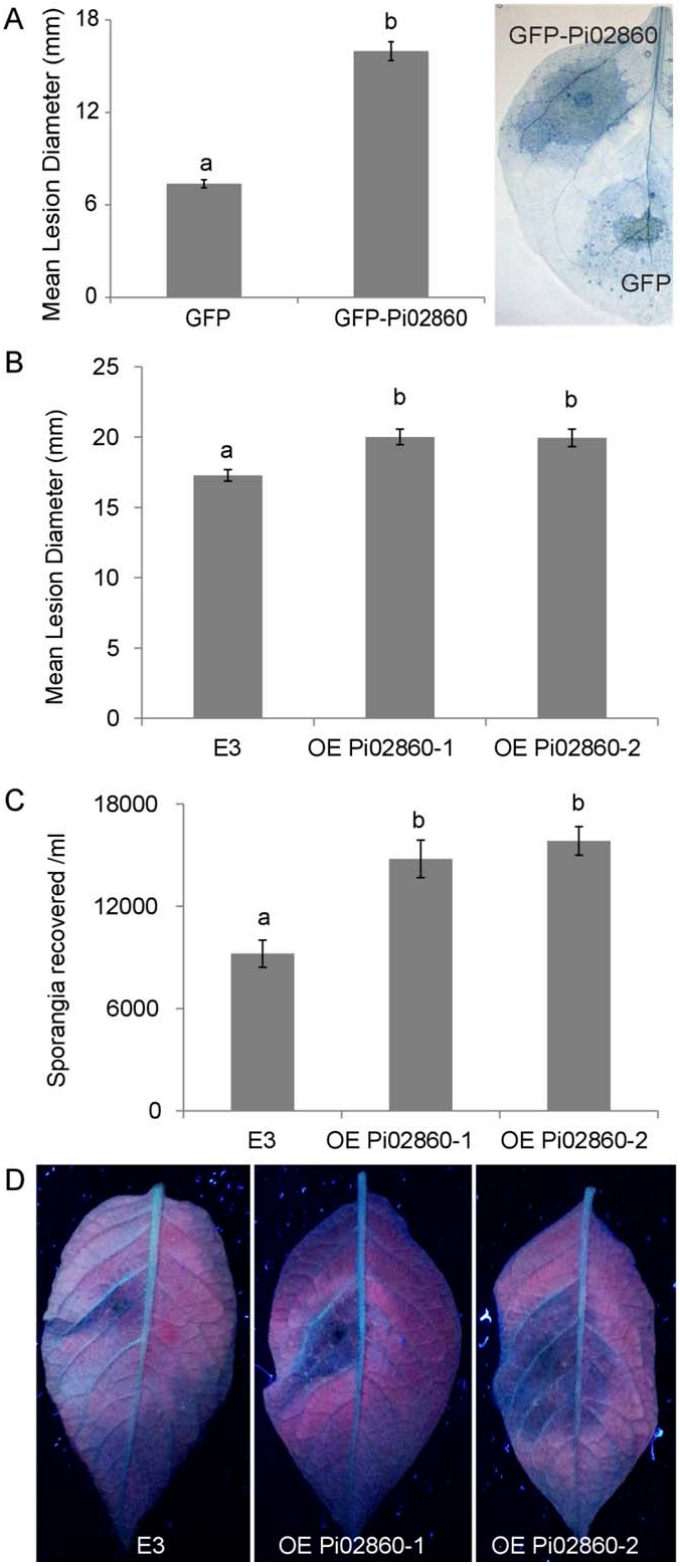
Here we show that either transient expression in model host *Nicotiana benthamiana*, or stable transformation in potato, of putative RXLR effector PITG_02860 (Pi02860) supports enhanced leaf colonisation by *P. infestans*. Expression of the effector in *N. benthamiana* also suppresses INF1-mediated cell death, indicating that Pi02860 contributes to PTI suppression. Pi02860 localises to the cytoplasm in *N. benthamiana*. Pi02860 interacts with an NPH3/RPT2-like (NRL) protein, StNRL1, in yeast-2-hybrid assays and *in planta*. Virus induced gene silencing (VIGS) of *NbNRL1* attenuates *P. infestans* colonisation and accelerates INF1-mediated cell death. Moreover, VIGS of *NbNRL1* prevents the ability of Pi02860 to suppress INF1-mediated cell death, whereas such suppression by AVR3a is unaltered, indicating that PTI suppression by Pi02860 is mediated by NRL1. In contrast, overexpression of StNRL1 alone enhances colonisation and suppresses INF1-mediated cell death, indicating that StNRL1 is a negative regulator of PTI and can thus be regarded as a susceptibility factor.

Results and Discussion

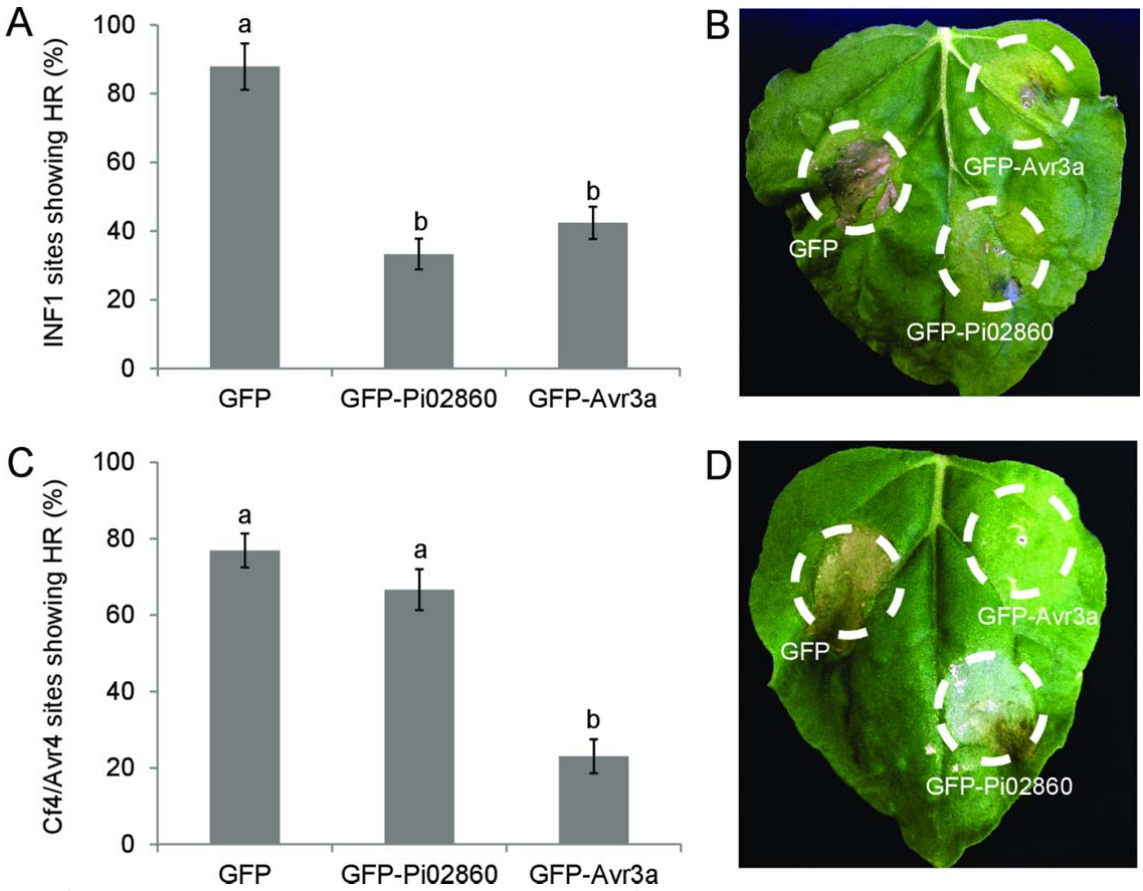
Pi02860 promotes *P. infestans* virulence and suppresses PTI

The effector Pi02860 (PITG_02860) is annotated in the *P. infestans* genome as a secreted RXLR type effector protein (Haas et al. 2009). Consistent with other RXLR effectors, *Pi02860* is specifically up-regulated at two and three days post-infection (dpi) of potato plants challenged with distinct *P. infestans* genotypes (Haas et al. 2009; Cooke et al. 2012). As these time-points correspond to the biotrophic phase of *Phytophthora* infection (Avrova et al. 2008) this effector was cloned and tested for its ability to influence *P. infestans* colonisation. A construct with GFP fused to the N-terminus of Pi02860 in place of the signal peptide was cloned and transiently expressed in the model solanaceous *P. infestans* host plant *N. benthamiana* using *Agrobacterium*-mediated expression followed by *P. infestans* challenge, as performed for other RXLR effectors (McLellan et al. 2013; Zheng et al. 2014; King et al. 2014). At 6 dpi significantly larger lesions (ANOVA, $p<0.001$) were observed in areas expressing GFP-Pi02860 compared to the expression of free GFP (Fig. 1A) thus suggesting that Pi02860 confers a benefit to the pathogen consistent with effector activity. To explore this phenomenon further in the host crop plant, transgenic potato lines were made for stable expression of Pi02860, minus signal peptide-encoding sequences (Supplemental Fig. S1). These plants were subsequently challenged with *P. infestans* and were also found to support significantly larger lesions (ANOVA, $p<0.002$) (Fig. 1B; 1D), and significantly enhanced sporulation (ANOVA, $p<0.001$) of the pathogen (Fig. 1C). The enhancement of *P. infestans* leaf colonisation promoted by Pi02860 expression inside host cells is similar to other recently described RXLR effectors (McLellan et al. 2013; Zheng et al. 2014; King et al. 2014; Wang et al. 2015; Boevink et al 2016) and consistent with it modifying the host to promote susceptibility.

As some RXLR effectors have been demonstrated to interfere with distinct defence signalling pathways *in planta* (e.g. Bos et al. 2010; King et al. 2014) GFP-Pi02860 was tested to determine if it attenuated cell death signalling activated by two characterised pathways. The *Phytophthora* PAMP INF1 triggers a hypersensitive response (HR) in some solanaceous hosts, including *N. benthamiana,* and this HR can be blocked by co-expressing RXLR effector AVR3a (Bos et al. 2010). In

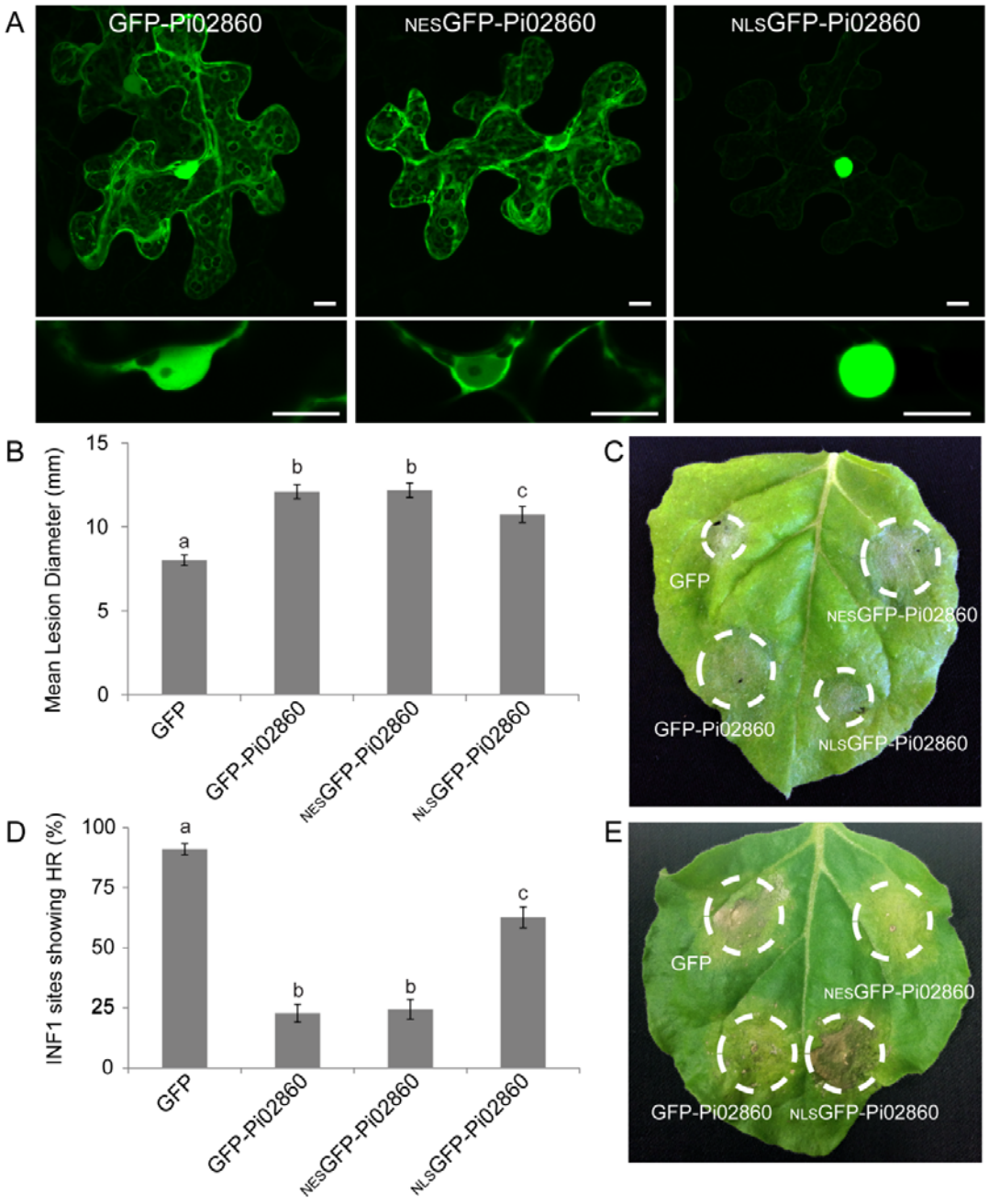


addition, effectors AVR3a and PexRD2 are both able to suppress the HR triggered by co-expression of the *Cladosporium fulvum* effector Avr4 and its cognate resistance protein Cf4 by different mechanisms (Bos et al. 2010; King et al. 2014). Expression of GFP-Pi02860 was found to significantly attenuate INF1-mediated HR



(ANOVA, $p<0.001$) to a similar level as the GFP-AVR3a control (Fig. 2A; 2B). In contrast to GFP-AVR3a, it had no significant effect on Cf4-CfAvr4 HR ($p>0.1$) (Fig. 2C; 2D). This suggests that the function of Pi02860 may be to suppress a specific signalling pathway(s) which is triggered on perception of *P. infestans* PAMPs, such as INF1, and does not extend to all cell death pathways triggered by activation of cell surface receptors.

To further investigate the phenotypes associated with Pi02860 overexpression the subcellular localisation of this protein was examined in *N. benthamiana* using confocal microscopy. GFP-Pi02860 was found to localise throughout the plant cytoplasm and nucleoplasm (Fig. 3A). To perturb the observed localisation of the effector two additional fusion constructs were generated, to which either a nuclear export signal (NES) or nuclear localisation signal (NLS) were added to the N-terminally fused GFP, as described previously (Wang et al. 2015). Both produced intact fusion proteins when expressed *in planta* (Supplemental Fig. S2). On examination with confocal microscopy, $_{NES}$GFP-Pi02860 was greatly reduced in the nucleoplasm but still accumulated in the cytoplasm, while $_{NLS}$GFP-Pi02860

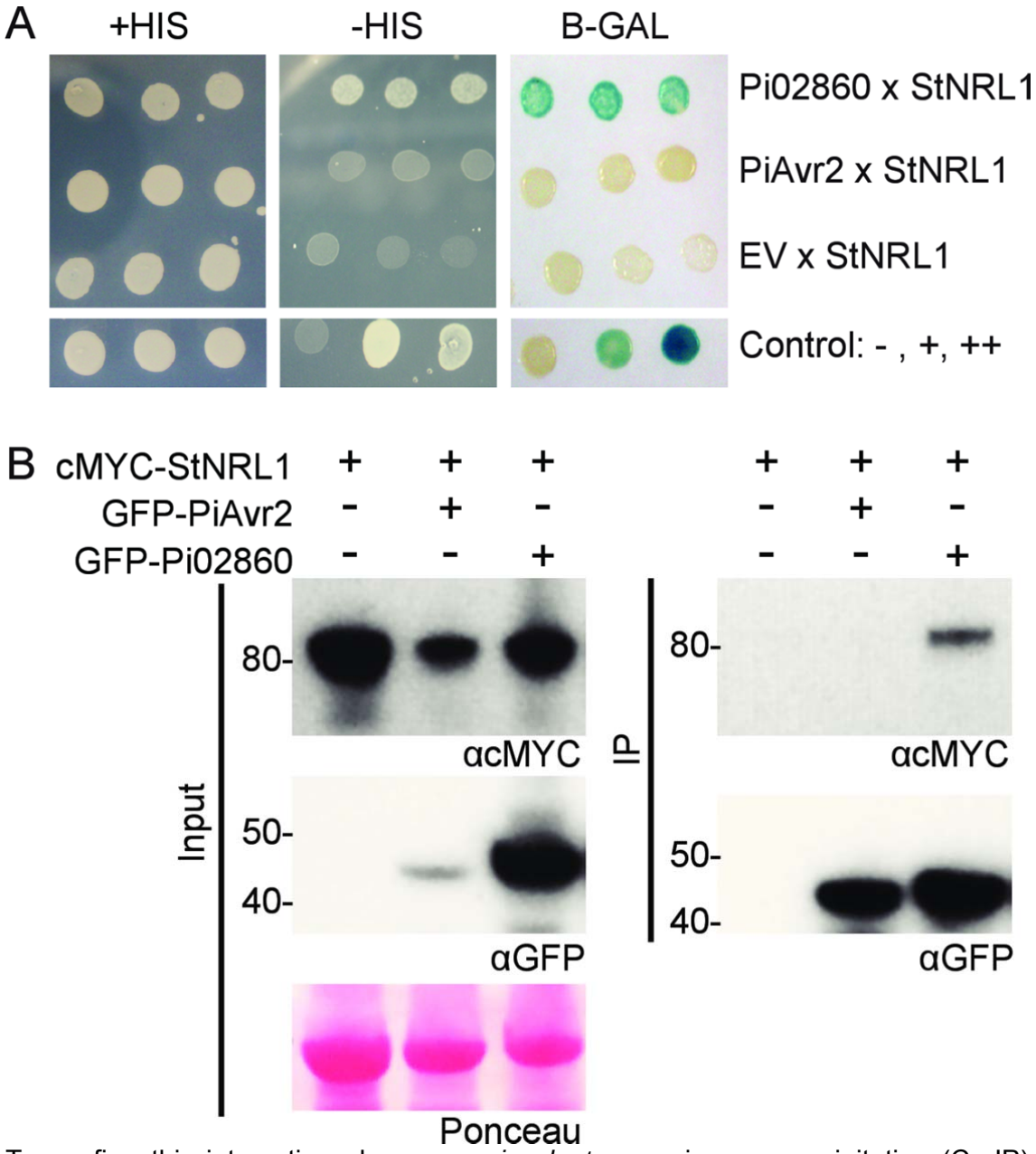


accumulated strongly in the nucleus and was largely reduced in the cytoplasm (Fig. 3A). The effects of these constructs were tested in the *P. infestans* virulence assay, using GFP-Pi02860 and GFP as positive and negative controls, respectively. Interestingly, expression of $_{NES}$GFP-Pi02860 was found to enhance leaf colonisation to the same level as GFP-Pi02860, while lesion sizes following expression of $_{NLS}$GFP-Pi02860 were significantly reduced compared to GFP-Pi02860 but were still significantly larger than free GFP (Fig. 3B; 3C). A similar pattern was observed when testing these constructs for their ability to suppress INF1-mediated HR. Again

199 $_{NES}$GFP-Pi02860 was found to suppress INF1 HR to similar levels as GFP-Pi02860.
200 In contrast, $_{NLS}$GFP-Pi02860 was significantly less able to suppress INF1-mediated
201 HR compared to GFP-Pi02860, but the HR was nevertheless more significantly
202 suppressed compared to that observed with free GFP expression (Fig. 3D; 3E).
203 Whilst the NES fusion did not totally exclude GFP-Pi02860 from the nucleus, and the
204 NLS fusion still retained background levels of cytoplasmic fluorescence, these
205 results may nevertheless indicate that the cytoplasmic localisation of Pi02860 is
206 more important for its contribution to virulence than the observed nucleoplasmic
207 localisation.

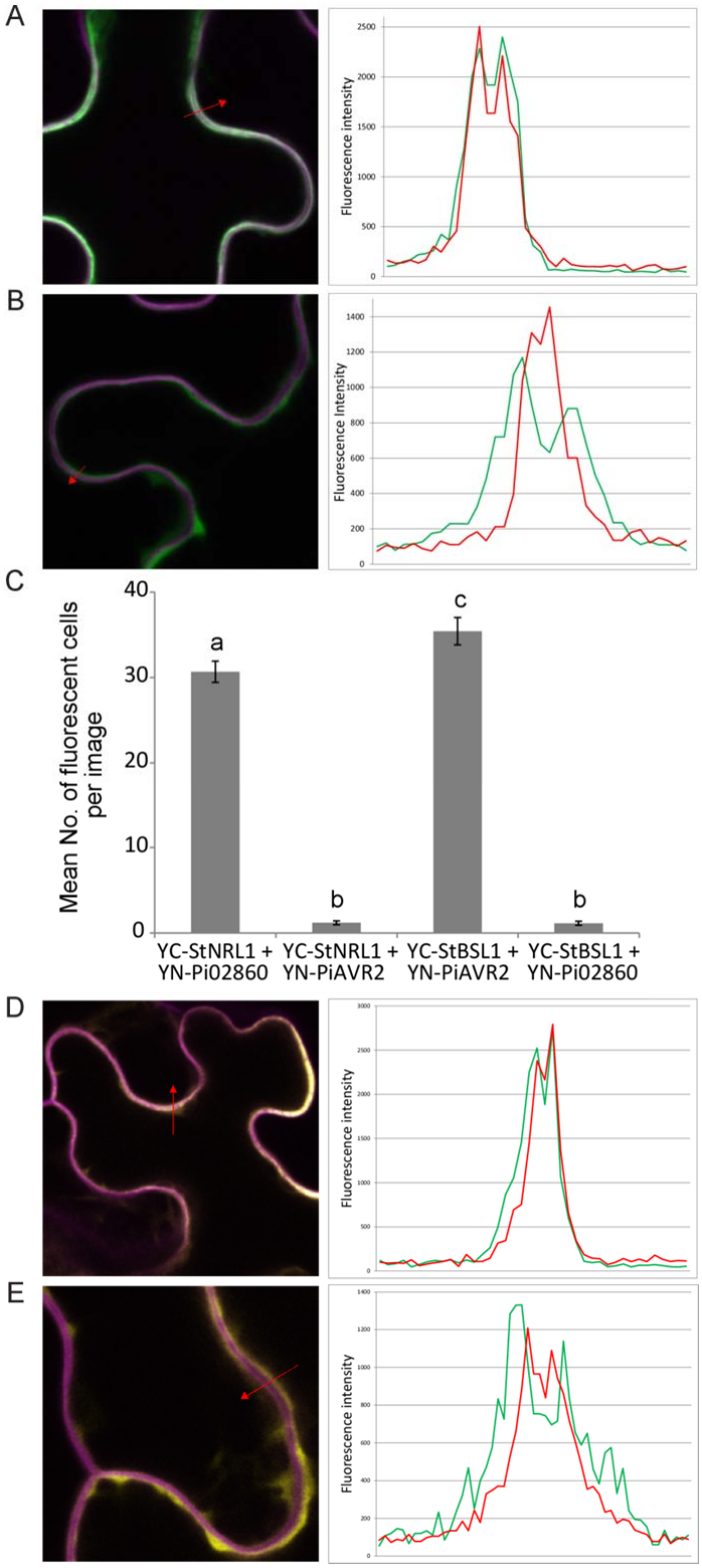
208 **Pi02860 interacts with the BTB/POZ domain protein StNRL1**

209 To further explore the mechanism of Pi02860 action in plants, a yeast-2-hybrid (Y2H)
210 library made from cDNA of potatoes infected with *P. infestans* (Bos et al 2010) was
211 screened with a GAL4 DNA binding domain-Pi02860 fusion ('bait') construct to a
212 depth of 0.44 x 10^6 yeast co-transformants. Five yeast colonies recovered from
213 selection plates that contained GAL4 activation domain ('prey') fusions, yielded
214 sequences corresponding to a potato Broad-Complex, Tramtrack and Bric-a-brac
215 (BTB/POZ) domain protein belonging to the Non-phototrophic hypocotyl 3/ Root
216 phototropism 2 (NPH3/RPT2)-Like family, hereafter referred to as StNRL1. In
217 Arabidopsis NPH3 and RPT2 interact with phototropins, mediating blue light
218 signalling, and are thought to be a core component of a cullin-3 (CUL3)-based
219 ubiquitin-protein ligase (E3) enzyme complex (Liscum et al. 2014). Supplemental
220 Fig. S3 shows an amino acid alignment of potato StNRL1 with its *N. benthamiana*
221 equivalents NbNRL1a and NbNRLb, the Arabidopsis protein At5g67385 (AtNRL) that
222 is a candidate orthologue (reciprocal best BLAST hit), and the characterised
223 At5g64330 (AtNPH3) and At2g30520 (AtRPT2), indicating the conserved domains
224 across these proteins. To confirm this interaction, a full length StNRL1 prey construct
225 was tested pairwise with bait constructs for Pi02860, a non-interacting RXLR control,
226 PiAVR2, which has been shown previously to associate with the putative
227 phosphatase BSL1 (Saunders et al. 2012), and the empty bait vector (EV). While all
228 transformants grew on the control plates (+ HIS) only Yeasts containing both
229 Pi02860 and StNRL1 were able to grow on the selection (–HIS) plates and activate
230 the β-galactosidase (B-GAL) reporter (Fig. 4A).



231 To confirm this interaction also occurs *in planta* a co-immunoprecipitation (Co-IP)
232 assay was performed by expressing cMyc-tagged StNRL1 (cMyc-StNRL1) alone or
233 with GFP-Pi02860 or GFP-PiAVR2 and pulling down with GFP-TRAP_M beads. Fig.
234 4B shows that, while all proteins were present in the relevant input samples, cMYC-
235 StNRL1 was only immunoprecipitated in the presence of GFP-Pi02860 and not alone
236 or with the GFP-PiAVR2 control.

237 To examine StNRL1 in more detail GFP was fused to its N-terminus to form GFP-
238 StNRL1 and was localised in *N. benthamiana* using confocal microscopy. The GFP-
239 StNRL1 fusion localised partially in the cytoplasm, but showed significant



accumulation at the plasma membrane (PM), when compared to a free GFP control (Supplemental Fig. S4). Co-expression of GFP-StNRL1 with an mOrange-LTi PM marker indicated significant co-localisation, which was not observed with free GFP

(Fig. 5A, 5B; Supplemental Fig. S4). This indicates that, whilst GFP-NRL1 is observed in the cytoplasm, it also strongly associates with the PM.

A bimolecular fluorescence complementation assay (commonly referred to as Split YFP) was then undertaken to establish the site of the interaction of StNRL1 and Pi02860 proteins *in planta*. The C-terminus fragment of YFP (YC) was fused to StNRL1 while the N-terminus (YN) was fused to Pi02860 to give YC-StNRL1 and YN-Pi02860, respectively. As the controls used in split YFP studies are important to rule out false positive interactions (Boevink et al. 2014) we used YC-StBSL1 and YN-PiAVR2 as controls for a plant target and effector interacting pair which also localise to the plant cytoplasm and PM (Saunders et al. 2012). Co-expression of either YC-StNRL1 with YN-Pi02860 or YC-StBSL1 with YN-PiAVR2 yielded fluorescence visualised by confocal microscopy, whereas there was no appreciable fluorescence when the YN-Pi02860 was co-expressed with YC-BSL1, or when YN-AVR2 was co-expressed with YC-NRL1 (Supplemental Fig. S4). This was quantified by counting the number of fluorescent cells in the field of view in >50 low magnification images each to show that YC-StNRL1 with YN-Pi02860 or YC-StBSL1 with YN-PiAVR2 gave significantly higher fluorescence (ANOVA, $p<0.001$) than YC-StNRL1 with YN-PiAVR2 or YC-StBSL1 with YN-Pi02860 (Fig. 5C).

The YC-StNRL1 and YN-Pi02860 constructs were co-expressed in *N. benthamiana* with the mOrange-LTi PM marker and YFP fluorescence was observed in the cytoplasm, but with significant accumulation at the PM using confocal microscopy, compared to free YFP control (Fig. 5D, 5E; Supplemental Fig. S4). The presence of each of the intact fusion constructs was confirmed by immunoblotting to rule out changes in fluorescence levels being caused by construct instability (Supplemental Fig. S5).

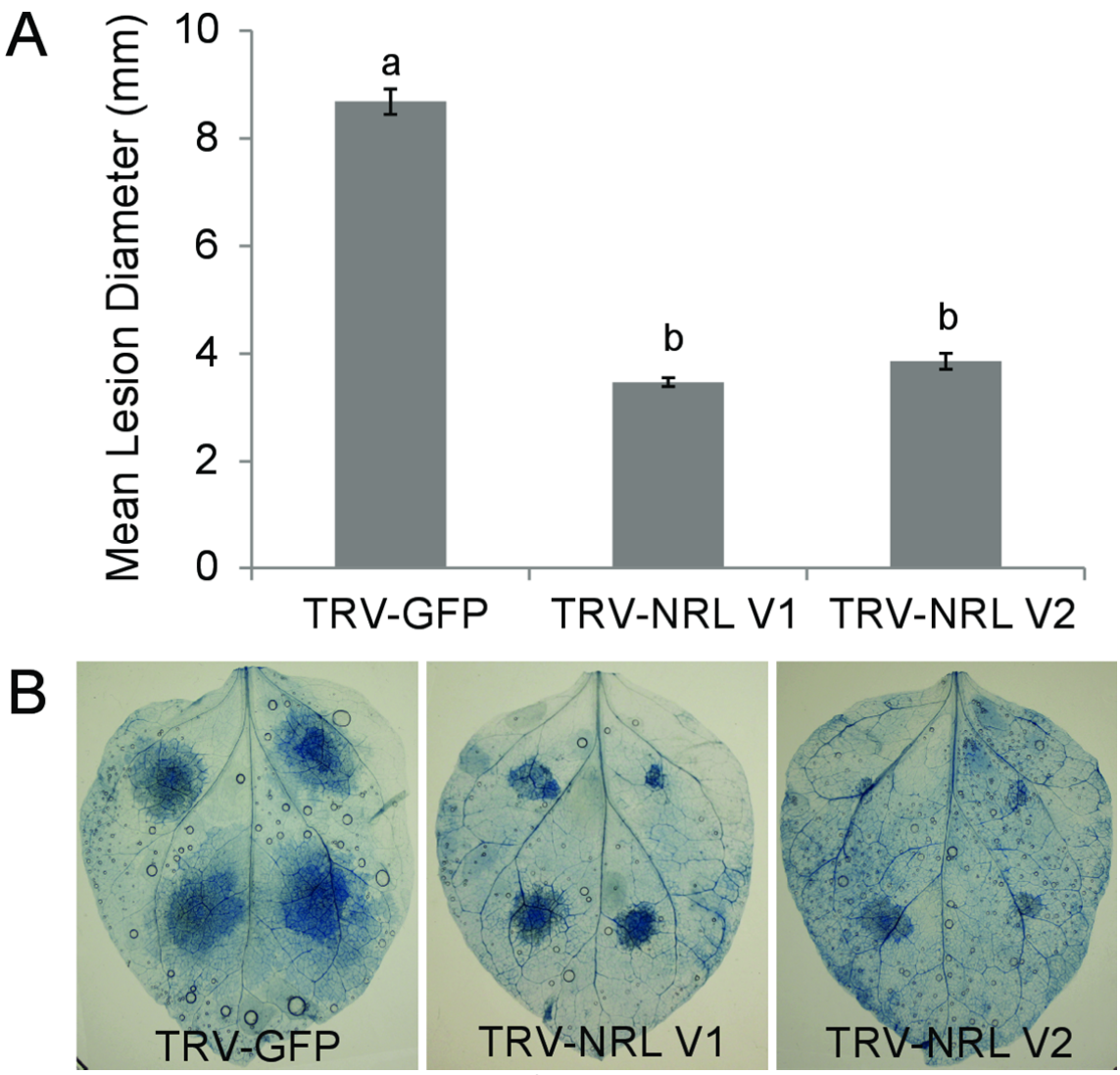
The localisation of StNRL1 at the host PM is in agreement with studies of NRL family members in Arabidopsis, all of which have been shown to interact at the PM, as part of a cullin-3 (CUL3) RING ubiquitin ligase (CRL) complex, with phototropins, which are involved in blue light signalling (Liscum et al. 2014).

StNRL1 silencing retards _P. infestans_ colonisation and prevents Pi02860 suppression of INF1-mediated cell death

To examine a possible role for StNRL1 in plant defence against *P. infestans*, virus induced gene silencing (VIGS) was used to knock down the expression of the equivalent *NRL1* genes in *N. benthamiana*. *N. benthamiana* is an allotetraploid and thus gene searches in the genome usually reveal two matching copies where the two homeologous genes have not been collapsed during assembly (Bombarely et al 2012). Consistent with that, two sequences, designated *NbNRL1a* and *NbNRL1b,* encoding proteins with 95 % amino acid identity to each other, were identified in the *N. benthamiana* genome. The predicted NbNRL1a and NbNRL1b proteins are each 84 % identical to StNRL1 (Supplemental Fig. S3). Consequently, two independent VIGS constructs, TRV-NRL V1 and TRV-NRL V2, were designed to silence both homeologous copies simultaneously. Supplemental Fig. S6A shows that transcript accumulation of both *NbNRL1a* and *NbNRL1b* are reduced by 60-85% in plants expressing either TRV-NRL construct, compared to plants expressing the TRV-GFP control. Representative images of plants expressing each TRV-NRL VIGS construct show that these plants exhibit a developmental phenotype, being stunted in growth compared to the TRV-GFP control (Supplemental Fig. S6B).

Following infection of the VIGS plants with *P. infestans* it was observed that silencing of *NbNRL1a* and *NbNRL1b* led to a reduction in the ability of the pathogen to colonise these plants, with significantly smaller lesions (ANOVA, $p<0.001$) developing on TRV-NRL plants compared to the TRV-GFP controls (Fig. 6A & 6B). This suggests that *P. infestans* requires the presence of NRL1 to establish normal infections, and would not support a model in which effector Pi02860 inhibits or inactivates NRL1.

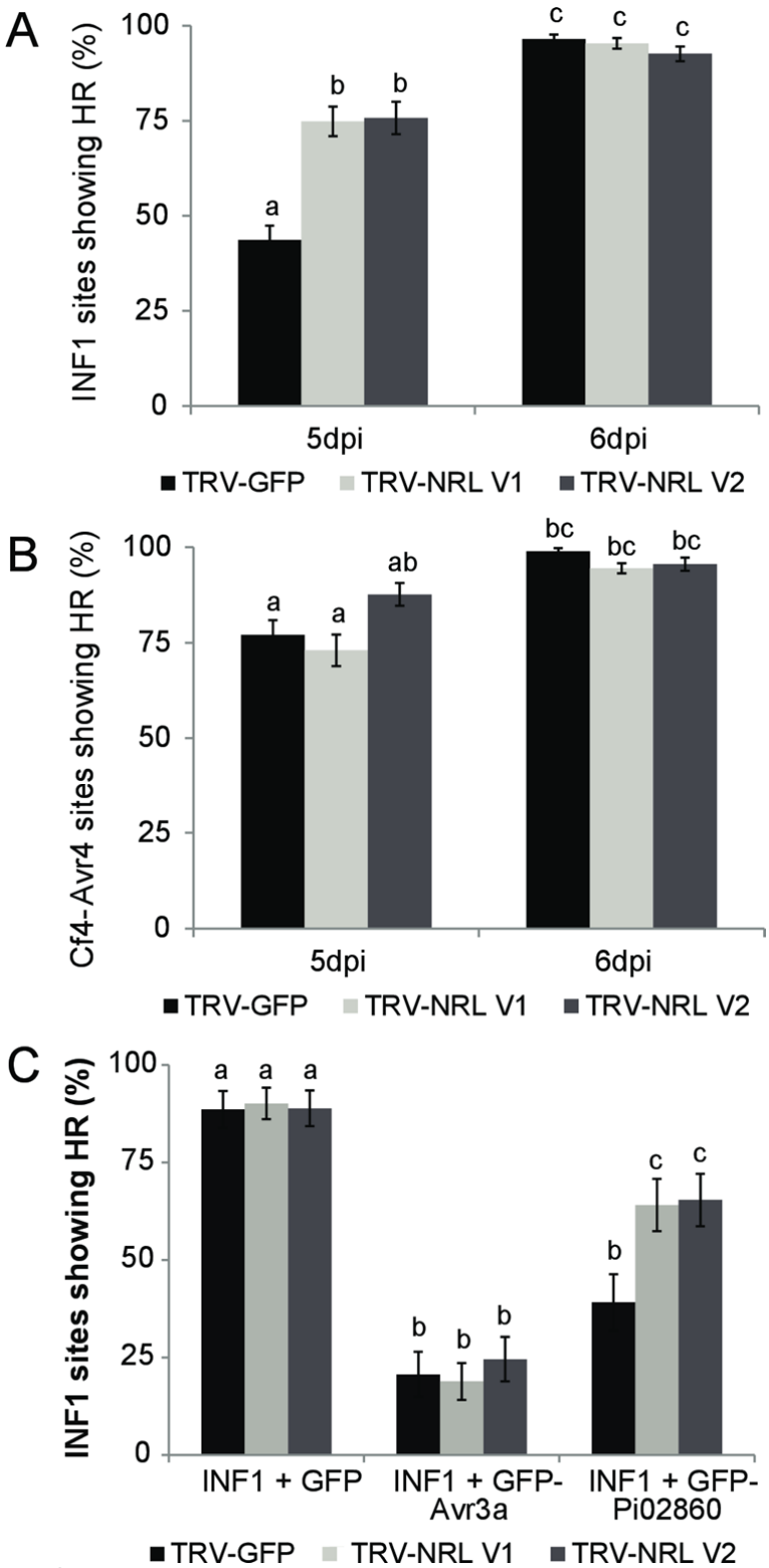
As Pi02860 was also observed to suppress INF1-mediated cell death, constructs expressing INF1 were agro-infiltrated into plants expressing each of the VIGS constructs and scored at 5 and 6 days post-inoculation (dpi). After 5 dpi a significant increase (ANOVA, $p<0.001$) in INF1 HR was observed on TRV-NRL VIGS plants compared to TRV-GFP (Fig. 7A). This difference was not apparent by 6 dpi indicating that the HR is accelerated by *NbNRL1* silencing. The same assay was carried out to examine the Cf4-Avr4 HR, which was not effected by Pi02860 expression. As anticipated, there were no significant differences in Cf4-Avr4 HR in TRV-NRL plants compared to TRV-GFP at either 5 or 6 dpi (Fig. 7B). These results suggest that NRL1 acts as a negative regulator of INF1-mediated cell death.



To investigate whether suppression of INF1-mediated cell death by Pi02860 is dependent on the presence of NRL1, either GFP-Pi02860 or, as a control, GFP-AVR3a, was co-expressed with INF1 in leaves expressing either the TRV-NRL VIGS constructs or TRV-GFP. Whereas GFP-AVR3a suppressed INF1-mediated cell

311 death to similar amounts on all plants, suppression of INF1-mediated cell death by
312 GFP-Pi02860 was retained on TRV-GFP plants, but was significantly reduced on
313 plants in which *NbNRL1a* and *NbNRL1b* were silenced (Fig. 7C). Some ability to
314 suppress INF1-mediated cell death was retained. However, this is likely due to the

315 fact that VIGS is notoriously ‘patchy’, with some leaf areas more efficiently silenced
316 than others, and that silencing knocked down the transcript levels of *NbNRL1a* and
317 *NbNRL1b* by 60-85 % (Supplemental Fig. S6), suggesting that some NRL1 protein is
318 likely present. Nevertheless, the significant reduction in INF1 cell death suppression



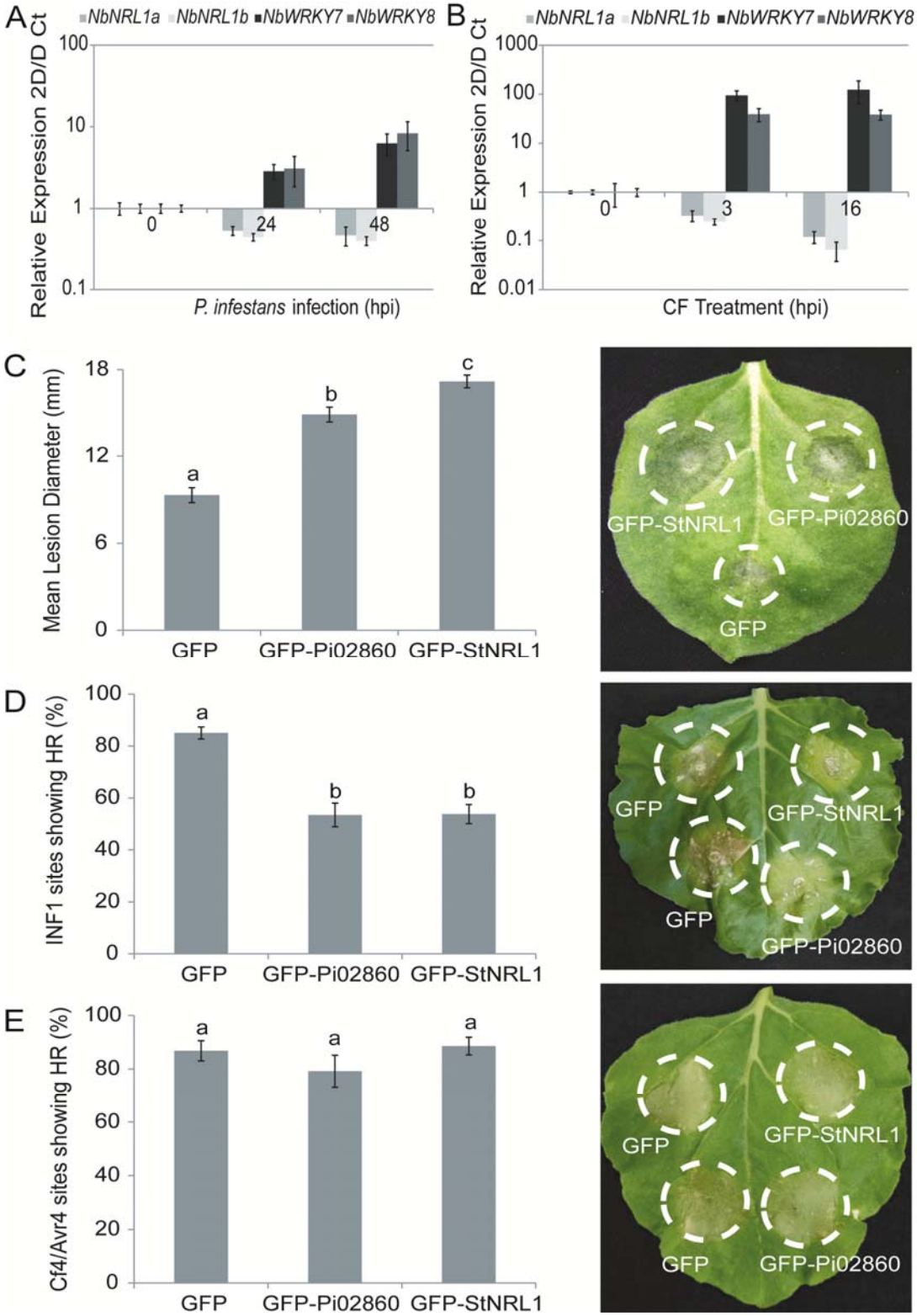
by GFP-Pi02860 provides a direct genetic link to indicate that this effector activity is dependent on the presence of NRL1. Taken together, the results indicate that NRL1 is a negative regulator of immunity and is thus unlikely to be inhibited by Pi02860.

StNRL1 overexpression suppresses INF1-mediated cell death and enhances *P. infestans* colonisation

Silencing of *NbNRL1a* and *NbNRL1b* by VIGS in *N. benthamiana* led to accelerated INF1-triggered cell death and reduced *P. infestans* colonisation, suggesting that NRL1 acts as a negative regulator of immunity. We thus investigated the expression of *NbNRL1a* and *NbNRL1b* during the first 48 hours of *P. infestans* colonisation of *N. benthamiana*, which can be regarded as the biotrophic phase of infection (Avrova et al 2008), and at times of 3 and 16 hours post-treatment with *P. infestans* culture filtrate (CF), which can be regarded as a cocktail of *Phytophthora* PAMPs (McLellan et al 2013). In contrast to two PTI marker genes, *NbWRKY7* and *NbWRKY8,* which were, similar to previous observations (McLellan et al 2013), weakly up-regulated during infection and strongly up-regulated by CF treatment, transcript accumulation of both *NbNRL1a* and *NbNRL1b* decreased weakly during infection and strongly with CF treatment (Fig 8A; 8B), indicating they are potentially down-regulated during immune responses. This is consistent with NRL1 acting as a negative regulator of immunity. To further investigate this we studied the effects of NRL1 overexpression.

Transient expression in *N. benthamiana* of either GFP-StNRL1 or GFP-Pi02860, followed by pathogen challenge was found to result in significantly larger *P. infestans* lesions ($p<0.001$) compared to free GFP expression, with GFP-StNRL1 overexpression having a larger infection-enhancing effect on *P. infestans* growth than GFP-Pi02860 (Fig. 8C). Moreover, either GFP-StNRL1 or GFP-Pi02860 expression independently suppressed INF1-mediated HR to a similarly significant level ($p<0.001$) compared to free GFP expression (Fig. 8D). In contrast, expression of either GFP-StNRL1 or GFP-Pi02860 had no significant effect on Cf4-Avr4-mediated HR (Fig. 8E).

Recently, the *P. infestans* effector Pi04089 has been shown to interact with a KH RNA binding protein, KRBP1, which is a susceptibility factor. KRBP1 protein turnover is reduced in the presence of Pi04089, suggesting that the effector enhances its stability (Wang et al 2015). We thus investigated whether such a phenomenon occurred with StNRL1 in the presence of Pi02860. However, in three independent replicates, GFP-StNRL1 protein stability was not enhanced by co-expression with cMYC-Pi02860, compared to a cMYC empty vector control (Supplemental Fig. S7).



This work shows that the *P. infestans* RXLR effector Pi02860, when expressed *in planta,* enhances pathogen colonisation and suppresses cell death triggered by perception of the *P. infestans* PAMP INF1. We show that it does this through its interaction with the potato BTB/POZ domain family protein StNRL1, with which it

interacts in the cytoplasm and at the plant plasma membrane, as silencing *NRL1* compromises the ability of Pi02860 to suppress INF1-mediated cell death. In contrast, *NRL1* silencing did not attenuate AVR3a suppression of INF1-triggered cell death, consistent with this effector acting through an alternative host target, the E3 ubiquitin ligase CMPG1 (Bos et al 2010). The observation that AVR3a retains its ability to attenuate INF1-mediated cell death on *NRL1* VIGS plants may suggest that AVR3a acts downstream of Pi02860 in suppressing this immune response.

Recently, functional redundancy in the *P. infestans* effector repertoire was highlighted by the demonstration that 8 out of 33 tested RXLR effectors were able to suppress early transcriptional responses to the bacterial PAMP flg22 (Zheng et al 2014). Of these 8 effectors, only 3 acted to suppress MAPK activation following flg22 treatment, indicating that this functional redundancy likely comprises different modes-of-action by these effectors; involving some acting upstream and others downstream of MAPK activation (Zheng et al 2014). The demonstration that Pi02860 and AVR3a (Bos et al 2010; Gilroy et al 2011) each suppress INF1-mediated cell death, but through activity on different targets, further emphasises functional redundancy comprising diverse effector activities.

NRL1 is a member of a family of proteins that include the functionally characterised NPH3 and RPT2, which interact with phototropins at the plasma membrane (PM) to mediate blue light signalling. The BTB/POZ domain in NPH3 promotes association with Cullin 3 (CUL3), forming a substrate adaptor in a $CRL3^{NPH3}$ (for Cullin-RING-ubiquitin-ligase) complex that targets phototropin phot1 for ubiquitination. High blue light conditions result in either mono-/multi- or poly-ubiquitination, the latter of which targets phot1 for degradation by the 26S proteasome, presumably to attenuate signalling under light-sufficient conditions (reviewed in Liscum et al 2014). Under low blue light conditions, in contrast, only mono-/multi-ubiquitination of phot1 occurs, which is necessary to establish phototropic responses (Roberts et al 2011). One of the consequences of phot1 activation by the combination of its phosphorylation and mono-/multi-ubiquitination, is its dissociation from the PM to stimulate relocalisation of PIN proteins from endosomes to the PM, where they facilitate auxin efflux (Liscum et al 2014). Poly-ubiquitination, targeting phot1 for proteasome-mediated degradation, would fail to re-localise PIN proteins and thus not stimulate auxin efflux.

Auxin is antagonistic to the defence hormone salicylic acid (SA), and increasing cellular levels of auxin is a strategy employed by numerous pathogens to suppress immunity (Naseem and Dandekar 2012). It is thus conceivable that Pi02860 could promote NRL1 activity, thus influencing phot1 levels, and therefore PIN re-localisation, retaining intracellular auxin levels to antagonise immunity. However, at this stage the function of the Arabidopsis orthologue of NRL1 is unknown, and it may not function similarly to NPH3, instead facilitating the ubiquitination and turnover of other proteins directly associated with immunity. To investigate this possibility, further work is needed to identify protein partners of StNRL1, including whether it forms a ubiquitin E3 ligase complex with CUL3.

A number of ubiquitin E3 ligases negatively regulate PTI, and these have been the subject of extensive functional studies. E3 ligases PUB12 and PUB13 both work to attenuate PTI by ubiquitinating the flg22 receptor FLS2, facilitating its degradation (Lu et al 2011). Recent yeast-2-hybrid (Y2H) screens have revealed potential co-regulatory partners and substrates for ubiquitination by PUB13, including phosphatidylinositol-4 kinase and RABA4B, with which it complexes to negatively regulate salicylic acid (SA)-mediated defences (Antignani et al 2015), and the ABA regulator ABI1, a PP2C family member, which is a PUB13 substrate for ubiquitination and degradation (Kong et al 2015). In addition, PUBs 22, 23, and 24 also suppress immunity. PUB22 attenuates PTI by targeting the exocyst component exo70B2 for ubiquitination and degradation (Stegmann et al 2012). A further example of E3 ligases that negatively regulate immunity are the BTB-domain proteins NPR3 and NPR4, which form complexes with CUL3 to facilitate the ubiquitination and degradation of the major SA regulator NPR1 in the nucleus (Fu et al 2012). If NRL1 forms a complex with CUL3 it may represent a further CUL3-based E3 ligase involved in negative regulation of immunity, albeit one that is predicted to function outside of the nucleus. Identification of its substrates for ubiquitination will reveal the mechanism underlying its defence suppression.

Whereas transient silencing of *NbNRL1*, using VIGS, accelerated INF1 cell death and attenuated *P. infestans* leaf colonisation, transient overexpression of StNRL1 resulted in the opposite phenotypes, indicating that, in the absence of the effector Pi02860, NRL1 is a negative regulator of immunity, and can thus be regarded as a susceptibility (S) factor. The term S factor has been coined to describe proteins with

a wide range of activities from cell wall alterations, to proteins that directly suppress or antagonise immunity, to those that provide metabolic changes of benefit to pathogen growth (van Schie and Takken, 2014). Many have been defined as such due to reduced pathogen colonisation when they are disabled, and/or increased disease development when they are overexpressed. Few such proteins have been demonstrated to be targeted by pathogen effectors. Examples include the *SWEET* genes that are induced by *Xanthomonas* TAL effectors, contributing to sugar efflux to provide pathogen nutrition (Chen et al. 2010); the *Pseudomonas* effector AvrB which mediates the phosphorylation and activation of MPK4, a negative regulator of PTI (Cui et al., 2010); and more recently, the *P. infestans* effector Pi04089, which targets and stabilises a KH RNA binding protein, StKRBP1, overexpression of which enhances susceptibility (Wang et al., 2015). Here we show that the target of Pi02860, NRL1, is an S factor that directly or indirectly suppresses PTI, in the form of INF1-mediated cell death. Future work will focus on how Pi02860 supports or promotes NRL1 activity, and in identifying the substrates and partner proteins of NRL1, and how it acts to enhance late blight susceptibility. Understanding how *P. infestans* can use endogenous host regulatory proteins and processes that may naturally undermine immunity will reveal novel means to control this pathogen. Further studies on NRL1, as a negative regulator of immunity, will indicate the mechanisms by which plants govern cross-talk between biotic stress responses and other cellular processes in an attempt to balance and allocate resources.

Materials & Methods

Vector construction

Phytophthora infestans putative RXLR effector gene Pi02860 was synthesised by Genscript with attL sites to generate an entry vector. To make overexpression vector PRI101-Pi02860 the effector was amplified from *P. infestans* cDNA with primers containing *Bam*HI and *Nde*I restriction sites and ligated into PRI101 using standard molecular biology techniques. The potato NPH3/RPT2-like protein StNRL1 coding sequence was amplified from *S. tuberosum* cDNA with flanking attB sites and PCR products were recombined into pDONR201 (Invitrogen) to generate entry clones using Gateway technology (Invitrogen) primer sequences shown in supplemental Table S1.

The effector entry clones were recombined with pDEST32 (for Y2H; Invitrogen), pB7WGF2 (for N-terminal EGFP fusion) (Karimi et al., 2002). Modified forms of pB7WGF2 with either an NES signal derived from PKI: amino acid sequence LALKLAGLDIN (Wen et al., 1995) or an NLS signal derived from SV40 T antigen: amino acid sequence PKKKRKV (Kalderon et al., 1984) added to the N-terminus of the GFP were created. The effector entry clones were also recombined with pCL112 (for N-terminal YN fusion) or pCL113 (for N-terminal YC fusion) for BiFC (Bos et al., 2010) and pGWB18 (for N-terminal tagging with the cMyc epitope), (Nakagawa et al., 2007).

Potato transformation

Agrobacterium containing overexpression vector PRI101-Pi02860 was used to transform microtuber discs of the potato cultivar E3 (Si et al., 2003; Tian et al., 2015). Positive lines were first screened on differential medium (3% MS+0.2 mg l-1 IAA + 0.2 mg l-1 GA3 + 0.5 mg l-1 6-BA + 2 mg l-1 ZT+75 mg l-1 Kan+200 mg l-1 Cef, pH 5.9) and then transferred to root generation medium (3% MS+50 mg l-1 kan + 400 Cef mg l-1, pH 5.9). The presence and expression level of the transgene was confirmed by semi-quantitative PCR (primers are shown in supplemental Table S1).

Plant production and maintenance

Nicotiana benthamiana and *Solanum tuberosum* overexpression (OE) lines were grown in glasshouses in 16h days at 22°C. Supplementary light was provided when the ambient light dropped below 200W / m2 and shading when it was above 450W / m2. Approximately five week old *N. benthamiana* and 7 week old *S. tuberosum* plants were used.

Agroinfiltration and infection assays

A. tumefaciens strain AGL1 transformed with vector constructs were grown overnight in YEB medium containing selective antibiotics at 28 °C, pelleted, resuspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$ and 200 µM acetosyringone) and adjusted to the required OD600 before infiltration into *N. benthamiana* leaves (generally 0.005 to 0.01 for imaging purposes, 0.002 for BiFC, 0.1 for infection assays and 0.5 for HR assays). For co-expression agrobacterial cultures carrying the appropriate vector constructs were mixed prior to infiltration.

Phytophthora infestans strain 88069 was used for plant infection and was cultured on Rye Agar at 19 ^{o}C for 2 weeks. Plates were flooded with 5ml H_2O and scraped with a glass rod to release sporangia. The resulting solution was collected in a falcon tube and sporangia numbers were counted using a haemocytometer and adjusted to 30000 sporangia/ ml, 10µl droplets were inoculated onto the abaxial side of leaves of intact *N. benthamiana* plants stored on moist tissue in sealed boxes. For VIGSed plants the average lesion diameter was measured and compared to the GFP control plants. *Agrobacterium tumefaciens* Transient Assays (ATTA) in combination with *P. infestans* infection were carried out as described (McLellan et al., 2013).

Confocal imaging

N. benthamiana cells were imaged at 2 dpi using Leica TCS SP2 AOBS, Ziess 710 or Nikon A1R confocal microscopes with Leica HCX PL APO lbd.BL 63x/1.20 W and L 40x/0.8, Zeiss PL APO 40x/1.0 or Nikon 60x/ water dipping objectives. GFP was excited by the 488 nm line of an argon laser and emissions were detected between 500 and 530 nm. The pinhole was set to 1 airy unit for the longest wavelength fluorophore. Single optical section images and z-stacks were collected from leaf cells expressing low levels of the protein fusions to minimise the potential artefacts of

ectopic protein expression. Images were projected and processed using the Leica LCS, Zen 2010 or NIS-Elements software packages. Subsequent image processing for Figure generation was conducted with Adobe Photoshop CS2 and Adobe Illustrator.

Yeast-two-hybrid and Co-immunoprecipitation

A Y2H screen with pDEST32-Pi02860 was performed as described in (McLellan et al., 2013) using the Invitrogen ProQuest system. The full-length coding sequence of the candidate interacting prey sequence, *StNRL1* (accession Sotub02g031050.1.1) was cloned and re-tested with pDest32-Pi02860 and pDEST32-PiAvr2 as a control to rule out the possibility that the observed reporter gene activation had resulted from interactions between the prey and the DNA binding domain of the bait construct or DNA binding activity of the prey itself.

A. tumefaciens strain GV3101 containing the fusion protein constructs were grown overnight in YEB medium containing selective antibiotics at 28 °C, pelleted, resuspended in infiltration buffer (10 mM MES, 10 mM MgCl2 and 200 µM acetosyringone) and adjusted to an OD600 of 1.0 before infiltration into *N. benthamiana* leaves. Forty eight hours post infiltration samples were taken and proteins extracted. GFP tagged Pi02860/ PiAvr2 fusions were immunoprecipitated using GFP-Trap®-M magnetic beads (Chromotek Gmbh). The resulting samples were separated by PAGE and Western blotted. Immunoprecipitated GFP fusions and co-immunoprecipitated c-Myc fusions were detected using appropriate antisera (Santa Cruz Biotechnology, UK).

Virus Induced Gene Silencing

Virus induced gene silencing (VIGS) constructs were made by cloning a 250 bp PCR fragment shared by *NbNRL1a* (accession NbS00004529g0005.1) and *NbNRL1b* (accession NbS00009404g0009.1) from *N. benthamiana* cDNA and cloning into pBinary Tobacco Rattle Virus (TRV) vectors (Liu et al., 2002) between *Hpa*I and *Eco*RI sites in the antisense orientation. BLAST analysis of this sequence against the *P. infestans* genome (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/ToolsIndex.html) did not reveal any matches that could initiate silencing in the pathogen. A

TRV construct expressing GFP described previously was used as a control (McLellan et al., 2013). Primer sequences are shown in Supplementary Table1. The two largest leaves of four leaf stage *N. benthamiana* plants were pressure infiltrated with LBA4404 *A. tumefaciens* strains containing a mixture of RNA1 and each NRL VIGS construct or the GFP control at OD600 = 0.5 each. Plants were used for assays or to check gene silencing levels by qRT-PCR 2-3 weeks later.

Gene expression assay

RNA was extracted using a Qiagen RNeasy Kit with on the column DNA digestion steps according to the manufacturer's instructions. First strand cDNA was synthesised from 2µg of RNA using Superscript II RNase HReverse Transcriptase (Invitrogen) according to manufacturer's instructions. Realtime qRT-PCR reactions were performed using Power SYBR Green (Applied Biosystems) and run on a Chromo4 thermal cycler (MJ Research, UK) using Opticon Monitor 3 software. Primer pairs were designed outside the region of cDNA targeted for silencing following the manufacturer's guidelines. Primer sequences in Supplementary Table1. Detection of real-time RT-PCR products, calculations and statistical analysis were performed as previously described (McLellan et al. 2013).

Supplementary Information

Figure S1. Transgenic potato lines overexpress Pi02860.

Figure S2. Immunoblots showing stability of the Pi02860 GFP fusions for re-localisation experiments.

Figure S3. Alignment of *Arabidopsis thaliana* (At), *N. benthamiana* (Nb) and *S. tuberosum* (St) NRL1 sequences.

Figure S4. Pi02860 interaction with NRL1 largely occurs at the plant plasma membrane.

Figure S5. Western blots showing stability of the different split YFP and GFP constructs used.

Figure S6. Silencing levels and plant phenotypes for VIGS of NRL1 in *N. benthamiana.*

Figure S7. Stability of StNRL1 is not altered by Pi02860.

Table S1. Primers used in this study.

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Figure Legends

Figure 1. Transient or stable overexpression of Pi02860 enhances *P. infestans* colonisation. A, Graph shows a significant increase ($p<0.001$, n=94) in *P. infestans* lesion diameter following transient expression in *N. benthamiana* of GFP-Pi02860 compared to a free GFP control. Trypan blue stained leaf image showing the extent of *P. infestans* colonisation with GFP-Pi02860 or free GFP. B, Graph shows that transgenic potato lines overexpressing Pi02860 (OE Pi02860-1 and OE Pi02860-2) allow a significant increase (ANOVA, $p<0.002$, n= 83) in *P. infestans* lesion diameter compared to the potato cv. E3 control. C, Graph shows transgenic potato lines overexpressing Pi02860 allow a significant increase ($p< 0.001$, n=64) in *P. infestans* sporulation compared to the E3 control. D, Leaf images taken under UV light show an increase in *P. infestans* lesions in transgenic potato lines overexpressing Pi02860 compared to the E3 control. Lower case letters on graphs denote statistically significant differences determined by one-way ANOVA with pairwise comparisons performed with the Holm-Sidak method. Results are the combination of at least two independent biological replicates, error bars show standard error.

Figure 2. Pi02860 supresses INF1 but not Cf4-Avr4 HR. A, Graph shows transient overexpression of GFP-Pi02860 compared to free GFP in *N. benthamiana* can significantly suppress the HR ($p<0.001$, n=11) triggered by the elicitin INF1 to a similar extent as the control GFP-Avr3a. B, Representative leaf image showing INF1 HR at 6 dpi, following co-expression with constructs as indicated. C, Graph shows transient overexpression of GFP-Pi02860 or free GFP in *N. benthamiana* show no significant difference ($p>0.1$) in HR triggered by CF4-Avr4 whereas GFP-Avr3a significantly suppresses this HR ($p<0.001$, n=13). D, Representative leaf image showing Cf4-Avr4 HR at 6dpi, following co-expression with constructs as indicated. Lower case letters on graphs denote statistically significant differences by one way ANOVA with pairwise comparisons performed with the Holm-Sidak method. Error bars show standard error.

Figure 3. Cytoplasmic localisation of GFP-Pi02860 is important for *P. infestans* virulence and INF1 HR suppression phenotypes. A, Confocal images showing that GFP-Pi02860 is localised in the cytoplasm and nucleus, while $_{NES}$GFP-Pi02860 is greatly reduced in nuclear fluorescence, and $_{NLS}$GFP-Pi02860 is concentrated in

609 the nucleus and reduced in the cytoplasm. Upper panels show stacked projections of
610 single cells, while the lower panels show single slice images of the nuclei. Scale bars
611 is 10 uM. B, Graph shows GFP-Pi02860 and $_{NES}$GFP-Pi02860 expression leads to a
612 statistically significant increase ($p<0.001$, $n=68$) in *P. infestans* lesion diameter
613 compared to free GFP, whereas $_{NLS}$GFP-Pi02860 shows an intermediate phenotype.
614 C, Representative leaf image showing *P. infestans* lesions following overexpression
615 of each construct, as indicated, in *N. benthamiana*. D, Graph shows GFP-Pi02860
616 and $_{NES}$GFP-Pi02860 co-expression with INF1 leads to a statistically significant
617 decrease ($p<0.001$, $n=41$) in HR compared to free GFP, whereas $_{NLS}$GFP-Pi02860
618 shows an intermediate phenotype. E, Representative leaf image showing INF1 HR
619 following co-expression with each construct, as indicated, in *N. benthamiana*. Lower
620 case letters on graphs denote statistically significant differences by one way ANOVA
621 with pairwise comparisons performed with the Holm-Sidak method. Results are the
622 combination of three independent biological replicates, each with 18 infiltration
623 zones. Error bars show standard error.

624 **Figure 4. Pi02860 interacts with the potato BTB/POZ domain protein StNRL1 in**
625 **yeast-2-hybrid and immunoprecipitation assays.** A, Yeast co-expressing StNRL1
626 with Pi02860 grew on -histidine (-HIS) medium and yielded β-galactosidase (B-Gal)
627 activity, while those co-expressed with the control PiAvr2 did not. The +HIS control
628 shows all yeast were able to grow in the presence of histidine. B,
629 Immunoprecipitation (IP) of protein extracts from agroinfiltrated leaves using GFP-
630 Trap confirmed that cMyc-tagged NRL1 specifically associated with GFP-Pi02860
631 and not with the GFP-PiAvr2 control. Expression of constructs in the leaves is
632 indicated by +. Protein size markers are indicated in kDa, and protein loading is
633 indicated by Ponceau stain.

634 **Figure 5. GFP-tagged StNRL1 predominantly locates to the plasma membrane**
635 **and Bi-molecular fluorescence complementation confirms StNRL1 and Pi02860**
636 **interaction.** A, Single optical slice image across plasma membranes (PM) of two
637 adjacent cells co-expressing GFP-StNRL1 and the mOrange-LTi PM marker with a
638 profile across the membranes in the location indicated by the red arrow. The plot of
639 the profile (right) indicates the majority of the GFP fluorescence (green line) co-
640 locates with the PM marker (red). B, A comparable profile in a cell co-expressing the
641 PM marker with un-fused GFP, which is only present in the cytoplasm. C, Graph

642 shows the average number of fluorescent cells per image with YC-StNRL1 + YN-
643 Pi02860 and YC-StBSL1 + YNPiAvr2 giving significantly more ($p<0.001$, $n=22$)
644 reconstitution of YFP fluorescence than when non-interacting effector-interactor pairs
645 are used. Lower case letters on graphs denote statistically significant differences by
646 one-way ANOVA with pairwise comparisons performed with the Holm-Sidak method.
647 Results are the combination of three independent biological replicates; error bars
648 show standard error. D, Single optical slice image across PMs of two adjacent cells
649 co-expressing YC-StNRL1 + YN-Pi02860 and the mOrange-LTi PM marker with a
650 profile across the membranes in the location indicated (arrow). The plot of the profile
651 (right) indicates the majority of the reconstituted YFP fluorescence (green line) co-
652 locates with the PM marker (red line). E, A comparable profile in a cell co-expressing
653 un-fused YFP in the cytoplasm with the PM marker.

654 **Figure 6. Silencing of *NRL1* in *N. benthamiana* compromises *P. infestans***
655 **infection.** A, Graph shows that silencing of *NRL1* using two independent VIGS
656 constructs (TRV-NRL V1 and TRV-NRL V2) in *N. benthamiana* significantly reduces
657 (one-way ANOVA, $p<0.001$, $n=464$; significance denoted by lower case letters) *P.*
658 *infestans* lesion diameter compared to the TRV-GFP control. B, Representative leaf
659 images stained with trypan blue to show the extent of *P. infestans* leaf colonisation
660 on plants expressing each VIGS construct, as indicated.

661 **Figure 7. Silencing of *NRL1* in *N. benthamiana* accelerates INF1 HR and**
662 **reduces the ability of Pi02860 to attenuate INF1 HR.** A, Graph shows a significant
663 increase ($p<0.001$, $n=30$) in INF1 HR in TRV-NRL V1 and TRV-NRL V2 plants
664 compared to the TRV-GFP control at 5 dpi but not at 6 dpi. B, Graph shows no
665 significant changes ($p>0.15$, $n=30$) in Cf4-Avr4 HR between TRV-NRL V1, TRV-NRL
666 and TRV-GFP at 5 or 6dpi. C, Graph shows that GFP-Pi02860 expression is
667 significantly less able to inhibit INF1 HR ($p>0.03$, $n=39$) in TRV-NRL V1 and TRV-
668 NRL V2 plants compared to TRV-GFP at 6dpi, while having no significant effect on
669 GFP-Avr3a INF1 HR suppression. Lower case letters on graphs denote statistically
670 significant differences by one-way ANOVA with pairwise comparisons performed
671 with the Holm-Sidak method. Results are the combination of three independent
672 biological replicates. Error bars show standard error.

673 **Figure 8.** ***NRL1*** **is downregulated by both** ***P. infestans*** **infection and PAMP treatment**
674 **while overexpression of NRL1 enhances** ***P. infestans*** **leaf colonisation and**
675 **suppresses INF1-triggered cell death.** A, Graph shows relative expression levels of
676 *NbNRL1a* and *NbNRL1b* and *NbWRKY7* and *NbWRKY8* in response to *P. infestans*
677 infection. B, Graph shows relative expression levels of *NbNRL1a* and *NbNRL1b* and
678 *NbWRKY7* and *NbWRKY8*in response to *P. infestans* Culture Filtrate (CF) treatment. C,
679 Graph shows that overexpression of GFP-Pi02860 and GFP-StNRL1 significantly
680 increases ($p<0.001$, $n=106$) *P. infestans* lesion size compared to free GFP.
681 Representative leaf image showing *P. infestans* lesions following overexpression of
682 each construct, as indicated, in *N. benthamiana*. D, Graph shows overexpression of
683 GFP-Pi02860 and GFP-StNRL1 significantly decrease ($p<0.001$, $n=44$) INF1 HR
684 compared to free GFP. Representative leaf image showing INF1 HR with
685 overexpression of each construct in *N. benthamiana*. E, Graph shows co-expression
686 of GFP-Pi02860 or GFP-StNRL1 have no significant effect ($P = 0.325$, $n=24$) on Cf4-
687 Avr4 HR compared to free GFP. Representative leaf image showing Cf4-Avr4 HR
688 with overexpression of each construct in *N. benthamiana*. Lower case letters on
689 graphs denote statistically significant differences by one-way ANOVA with pairwise
690 comparisons performed with the Holm-Sidak method. Results are the combination of
691 three independent biological replicates. Error bars show standard error.

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